SEROLOGICAL RELATIONSHIPS AMONG ORTHOPTEROID INSECTS AS DETERMINED BY THEIR WHOLE "BLOOD"[1]

BY LUDWIG K. PAULY

ZOOLOGY DEPARTMENT, UNIVERSITY OF WISCONSIN IN MILWAUKEE

Although precipitins were discovered in 1897 and various techniques have been employed for determining the strength of precipitin reactions, relatively few taxonomists have used this serological reaction in the study of animal relationships. This approach to the study of animal relationships showed great promise for clarifying disputed or undetermined relationships as early as 1904 when Nuttall published a book summarizing the results of some 16,000 tests in which some thirty different antisera were used on 900 species of animal blood. These results, in general, paralleled the existing classification of these animals as based on morphological and embryological characteristics. The solution of taxonomic problems has been carried out by means of the precipitin reaction (Graham-Smith on the king crab, Eisenbrandt on worms, and Wilhelmi on the phylogeny of the Chordates) but, all in all, there has been a general lack of use of precipitins for settling relationship disputes.

The class Insecta, being the largest class of animals in the animal kingdom, naturally has its share of members with uncertain taxonomic position. There is a general agreement in the gross picture of insect classification, but, when it comes to the relationship of orders to each other and families in each order, each author seems to have his own idea of classification. There is, perhaps, no better example of this variation in classification than among the orthopteroid insects. Comstock (1933), for instance, includes the families Tettigoniidæ, Gryllidæ, Locustidæ, Phasmidæ, Mantidæ and Blattidæ in the order Orthoptera and differentiates between the jumping forms (Saltatorial Orthop-

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tera) and the walking forms. Ross (1948), on the other hand, divides the order Orthoptera into four suborders, Blattaria, Mantodea, Phasmida, and Saltatoria. Essig (1942) has still a different classification for the orthopteroid insects. He places the saltatorial forms in the order Orthoptera and each of the walking forms in separate orders: Blattaria, Phasmida, and Mantodea. Finally Blatchley (1920) has still a different classification. He divides the Orthoptera into four suborders: Dermaptoria, containing the family Forficulidæ, the earwigs; Cursoria (runners) including the family Blattidæ; Gressoria (walkers), including the families Mantidæ and Phasmidæ; and Saltatoria (jumpers), including the families Tetrigidæ (pygmy locusts), Acrididæ, Tettigoniidæ and Gryllidæ.

All of these authors base their classification on certain morphological features and each author expresses his own opinion by his classification. Thus we have variations in classification. These discrepancies in classification could be avoided with a more objective basis for classification.

No one can deny that the basic nature of any organism is found in its biochemical constitution. The best known means of measuring the biochemical relationship of different organisms is the precipitin testing technique. As Wells (1930) so aptly puts it, "The serologic reactions are essentially delicate methods for differentiation of proteins (either alone or as the complexes existing in protoplasm), and since the evolution of different species is essentially the evolution of different combinations of amino-acids to form the proteins characteristic of each species, . . ., evidently the serological reactions afford a means for the analysis of the evolutionary relationship of species."

Several serological studies on insects have been carried out in the past. Brown and Heffron (1928) and Martin and Cotner (1934) on certain Leidoptera, Cumley (1940) on Diptera and Leone (1947) on Orthoptera have all carried out successful studies in this respect. Brown and Heffron and Martin and Cotner used the ring test technique, Cumley used the ring test technique and complement-fixation, and Leone used the ring test technique and the Libby photronreflectometer (1938). All of these authors obtained their insect antigens by saline extractions from ground-up insect bodies. This is a questionable prac-

tice since Canning (1929) showed that different organs in the same species showed some differences in serological reactions.

In serological studies with larger animals it is customary to use the blood serum as the antigen. In this paper the author has made an attempt to continue this practice with insects. Naturally, only insects of relatively large size can be used.

MATERIALS AND PROCEDURE

Whole "blood" was obtained from each of the following species:

Order ORTHOPTERA

Family	*Scientific Name*	*Common Name*
Blattidæ	*Periplaneta americanum*	American cockroach
	Blatta orientalis	Oriental cockroach
	Leucophea maderiæ	Tropical roach
Acrididæ	*Romalea microptera*	Florida lubber grasshopper
	Melanoplus femur-rubrum	Red-legged grasshopper
Mantidæ	*Paratenodera sinensis*	Chinese praying mantis
Phasmidæ	*Anisomorpha* sp.	Walking stick

Order COLEOPTERA

Scarabæidæ	*Phyllophaga* sp.	May beetle

Some of the insects used in the tests were either raised or collected by the authors, while others were obtained through various biological supply houses.

Special "syringes" were made for bleeding the insects. Glass tubing seven millimeters in diameter and approximately twenty centimeters long was heated through the middle and drawn into long slender, tapering tubes. When these tapering tubes were broken at the narrowest point and a rubber bulb from an eye dropper attached to the blunt end, an excellent instrument for bleeding insects was obtained.

The insects were starved for at least twenty-four hours and were then anaesthetized for bleeding. The slender tapered end of the syringe was inserted through the softer tissue between

the first and second tergum into the dorsal vessel of the insect. By careful manipulation of the rubber bulb a small amount of "blood" was obtained from each insect. Great care was necessary in inserting the syringe because of the delicate nature of the dorsal vessel. The tropical roach (*Leucophea maderiæ*) was found to yield the largest amount of blood per individual. Fifty of these insects would yield an average of 3.5 to 5 ml. of blood.

The blood of the various species of insects bled varied in color from a watery, colorless liquid as found in some of the cockroaches to a beautiful blue-green liquid as found in the Chinese mantids. The color of blood varied not only from species to species but also varied considerably from individual to individual in the same species.

The blood obtained from any one series of bleedings was pooled and allowed to stand for several hours—usually over night in a refrigerator. Several of the bloods formed a sort of clot while others did not. All of them, however, did have a fatty residue after standing for a time. The blood of the Florida lubber grasshopper (*Romalea microptera*) and the walking stick (*Anisomorpha* sp.) turned a dark almost black color after a few hours.

The blood was then centrifuged and the serum was stored in sterile vials in a freezer for future use. No attempt was made to determine protein content of the sera at this time.

Both chickens and rabbits were used for the production of antisera. For the ring test chicken antisera were used exclusively. Each chicken was given a single intravenous injection of 1 ml. of 2% dilution of the insect serum used as antigen. Wolfe (1942) found that this injection procedure usually yielded specific antiserum of high titer. Seven days after the injection the chickens were starved for 24 hours and bled completely by cardiac puncture. This blood was allowed to clot at room temperature. The clot was broken up and the blood was centrifuged. The antiserum was poured off and stored in cotton stoppered containers in the refrigerator for at least eight days. It was shown (Wolfe and Dilks, 1946) that the rise in titer of chicken antiserum occurred on standing and this was usually at a maximum after this length of time. Then the antiserum was used immediately or filtered and stored in sterile, rubber-stoppered vials in the refrigerator.

The ring test technique was performed similar to that of Boyden (1926) except that the original antigen dilution was a 2% solution of the insect's serum. Readings were taken at 5, 10, 20, 30 and 60 minute intervals, but only the 60 minute readings are recorded in this paper. Proper care was taken in layering the antiserum under the antigen and handling the tubes in order to maintain a clear interface.

A photoelectric instrument which has never been used in any serological study of taxonomy is the microdensitometer of Baier (1943). This instrument measures the amount of light transmitted by a turbid medium. It can cover an extremely wide range of turbidity concentration without readjustment but is less sensitive than certain other photoelectric instruments and consequently requires a much stronger antiserum for taxonomic studies.

In order to build up a strong enough antiserum for testing with the microdensitometer, a multiple series injection technique was employed. A rabbit was given a multiple series of injections of undiluted serum of the tropical roach (*L. maderiæ*). The first series consisted of injections of 0.5 ml., 1.0 ml., and 1.5 ml. of the roach serum into the lateral ear vein on alternate days. Thirty days after the last injection this series was repeated. Seven days after the last injection of the second series the rabbit was starved for 24 hours, anaesthetized, and bled completely by cardiac puncture. The blood was allowed to clot at room temperature. The clot was broken up, the blood was centrifuged, and the antiserum was filtered and stored in sterile, rubber-stoppered serum vials for future use.

The microdensitometer tests were conducted according to the procedure as outlined by Baier (1947). The tubes used in these tests were standardized according to diameter and transmittancy of light beams. This means that each tube in any one set of tubes would, when filled with distilled water, transmit the same amount of light in the instrument as any other tube in that set.

The microdensitometer readings should be made with antigen dilutions so chosen that the final readings for the highest and lowest dilutions (antibody excess and antigen excess) should equal the control reading at those two points. This was not possible at the antigen excess level because of the small amount of

antigen and the relatively low protein content of the insect's sera. Protein N determinations were made when sufficient antigen was available and 2% standard solutions containing 0.16 gms. N or protein per 100 ml. were prepared.

These 2% standard dilutions were used in the initial tube in the microdensitometer readings. As will be noted (Figure 1)

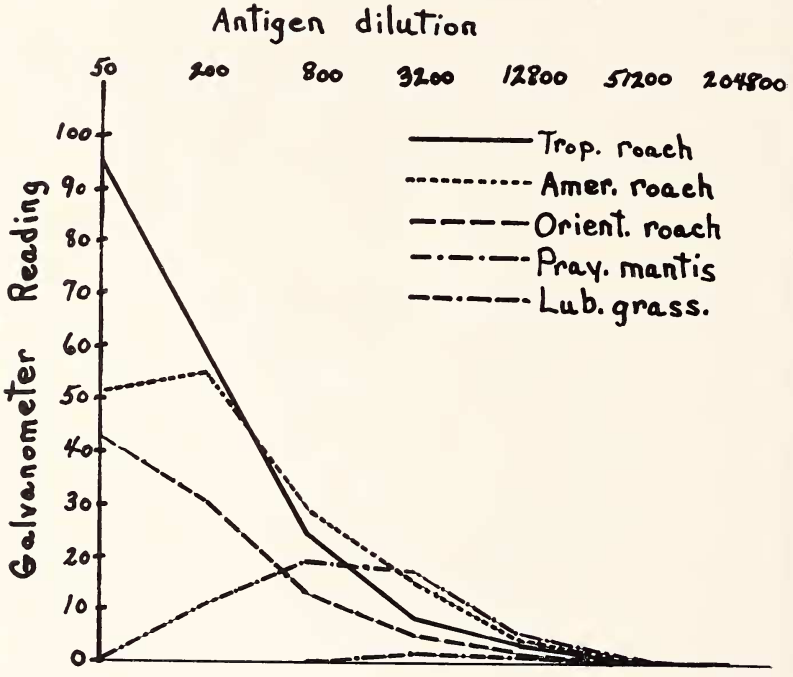

Figure 1

this procedure failed to produce readings in the prozone of the antigen-antibody reaction curve in some cases. In order to conserve antiserum only alternate tubes were used in the tests. No duplicate tests were run.

EXPERIMENTAL RESULTS

Successful antisera were produced against the tropical roach (*Leucophea maderiæ*), the lubber grasshopper (*Romalae microptera*), and the walking stick (*Anisomorpha* sp.). The limited amount of antigen prevented attempts to produce others.

Table I presents a summary of the results obtained with various antisera using the ring test technique and the microdensitometer. The first two anti-tropical roach sera were the same pooled immune serum obtained by combining the serum from several chickens. This combined serum was filtered and stored in sterile

TABLE I

RING TEST AND MICRODENSITOMETER RESULTS

Antisera	Homologous titer in hundreds	Antigens (Relationship values in per cent)							
		Tropical roach	Oriental roach	American roach	Walking stick	Chinese mantis	Lubber grasshopper	Red-legged grasshopper	May beetle
Tropical roach P.C.1—1949	128	100	25		12.5		3.1	0	
Tropical roach P.C.1—1950	64	100	12.5		1.6		0	0	
Tropical roach 41	256	100	12.5	.4	25	0	1.6	3.1	0
Lubber grass. (pool.) P.C.2	128	0	1.6	0	0	0	100	12.5	0
Lubber grass. 2876	128	25	1.6		0		100	50	
Walking stick (pool.) P.C.3	64	0	0	1.6	100	0	1.6	1.6	0
Tropical roach JD—diluted 1 + 2	128	100	50	100	0	100	.4		
JD—microdens		100	44.1	82.3	.3	27.7	1.3		

Note: First six tests run with non-standardized antigens. Last two run with 2% standard antigens.

vials in the refrigerator for a year. It is interesting to note in this pooled antiserum that the interfacial titer remained constant but the specificity seemed to increase during the first year. On the basis of these tests the tropical roach shows a closer relationship to the oriental roach than to any other insect tested. A relatively strong cross-reaction was obtained between the anti-tropical roach serum (1949) and the walking stick serum and a slight reaction was obtained with the lubber grasshopper serum.

Serum 41 against the tropical roach gave results that were similar to those obtained from the pooled tropical roach sera. It is interesting to note that again there is a weak reaction with the sera of the two jumping forms of Orthoptera tested.

For reciprocal tests three different antisera were used, two against the lubber grasshopper and one against the walking stick. Of the two antisera against the lubber grasshopper the pooled antiserum was again more specific. In testing this pooled antiserum only two heterologous reactions were obtained, a relatively strong one against the red-legged grasshopper and a very faint one against the oriental roach.

Antiserum 2876 against the lubber grasshopper showed a strong heterologous reaction against the red-legged grasshopper and the tropical roach and a very faint one against the oriental roach. This antiserum was definitely less specific than the pooled antiserum which probably accounts for the strong cross-reaction with the tropical roach.

The walking stick antiserum was a pooled antiserum of a very specific nature. Weak heterologous reactions were obtained with the American roach, the lubber grasshopper and the red-legged grasshopper.

All of the above tests were run with non-standardized antigens. This combined with the fact that the protein contents of the various insect sera varied considerably might lead one to believe that these tests were not too trustworthy.

The last two tests in Table I were run with 2% standard antigens. The antiserum in both of these tests was from the same rabbit. The results of the microdensitometer test are shown in Table I—last line. With the microdensitometer heterologous reactions were obtained with all antigens tested. On the basis of this test the American roach showed a much closer relationship to the tropical roach than did the oriental roach. The Chinese mantis showed a surprisingly close relationship to the tropical roach although it is considerably more distant than any of the roaches are to each other. The lubber grasshopper and walking stick showed only a slight relationship to the tropical roach.

Using one part of this same antiserum diluted with two parts of buffered saline, ring tests were performed with the same antigens used in the previous test. The results paralleled those of

the microdensitometer although no distinction could be made between the tropical roach, the American roach and the Chinese mantis. No cross reaction was obtained with the walking stick and only a very faint reaction was obtained with the lubber grasshopper.

DISCUSSION

The above results combined with those of Leone are at best only a crude beginning in the serological study of orthopteroid relationships. The insect species used in this study were chosen because of their large size and availability. This study was made with two purposes in mind: (1) to show the possibility of performing serological tests using the blood serum of insects rather than the extracts of whole insects or parts thereof; and (2) to attempt to give a serological basis for the taxonomic classification of the orthopteroid insects.

To the best of the authors' knowledge this is the first serological study of insects in which the whole blood sera of insects is used rather than saline extracts of the macerated bodies of insects. In the serological studies of all of the Chordata and many of the Invertebrata whole blood sera were used as the antigen. Only in cases where the animals studied were too small or where they lacked a well-defined circulatory system was it found necessary to use a saline solution to extract the body protein. The validity of the use of saline extracts of whole organisms in establishing relationships has been frequently discussed but never proven. Leone (1947) conducted serological studies of the Orthoptera using saline extracts for antigens. The results of this study are quite similar to those of Leone. More work should be done comparing results obtained by using whole blood sera with those obtained by using saline extractions. If these results are parallel at all times then—and only then—can the use of saline extracts of whole organisms in establishing relationships be considered valid.

As indicated previously each author seems to have his own classification for the orthopteroid insects. On the basis of the serological data obtained in this study indications are that all orthopteroid insects belong in the single order, Orthoptera. In no case was any cross reaction obtained when orthopteroid antisera were tested with serum from the May beetle, a member of

the order Coleoptera. With the sera of each of the orthopteroid forms, however, at least a slight reaction was obtained in one or more of the tests carried out.

In order to determine whether the order Orthoptera should be further divided into four suborders (Ross) or whether this order should merely contain six families (Comstock), more tests especially of the reciprocal nature, would have to be carried out. This author hopes to collect more insect sera and carry out these tests in the not too distant future.

SUMMARY

Serological tests were carried out using the whole blood serum of seven orthopteroid insects and one coleopteran insect.

Comparisons were made between results obtained from the "ring" test and the Baier microdensitometer.

On the basis of the results obtained the roaches showed a closer relationship to the Chinese mantis and the walking stick than to the jumping forms tested.

The results suggested that the orthopteroid insects belong in a single order rather than three or four different orders.

The right to accept results obtained by using saline extractions for antigen was questioned and a solution was suggested.

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(*Continued from page 82*)

Becker. On the serious side he pointed out the importance of the airplane in the transportation of insect and other epidemic producing pests, among them the Golden Nematode. Mr. Becker concluded that the greatest difficulty and hazard of plant quarantine inspection work is not with insects but with *Homo sapiens*.

The meeting adjourned at 9:30 P.M.

LOUIS S. MARKS, *Secretary*

MEETING OF MARCH 17, 1953

The meeting of the Society was called to order at 8:00 P.M. by the President, Dr. Lucy Clausen. In the absence of the Secretary, Mr. Marks, the minutes of the previous meeting were not read and the President asked Dr. James Forbes to serve as Secretary for the evening. There were 15 members and 7 guests present. Dr. Clausen cordially welcomed the guests to our meetings. She also greeted two members, Dr. Harold Hagen and Mr. Edwin W. Teale, who have not been with us recently. This was Dr. Hagan's first meeting in several months since his recent illness. Mr. Teale had just returned from a 28,000 mile, six-month jaunt around the country.

Mr. Jay Fox of Seaford, New York, was proposed for membership.

(*Continued on page 94*)